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Purification of Hemoglobin from the Actinorhizal Root Nodules of *Myrica gale* L.[1]

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Hemoglobins are generally absent or present in low concentrations in the nodules of actinorhizal plants. An exception is *Casuarina*, where a hemoglobin occurs at relatively high concentration. However, this plant is unique in that *Frankia*, the microsymbiont, lacks the vesicles that are normally the site of nitrogen fixation. The present paper shows that a hemoglobin also occurs at high concentrations in *Myrica gale* L., an actinorhizal plant in which *Frankia* does form vesicles. Hemoglobin was extracted from root nodules under anaerobic conditions using a buffer containing CO, detergent, and a reducing agent. Carboxyhemoglobin was purified using gel filtration followed by aerobic ion-exchange chromatography. The optical absorption spectra of the oxy-, deoxy-, and carboxyhemoglobins were similar to those of other hemoglobins. The molecular mass of the native hemoglobin estimated by gel filtration was 38,500 D. The molecular mass of the subunits estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis was 16,200 D, consistent with the mass of other hemoglobin subunits. Thus, the native hemoglobin is probably a dimer.

Hemoglobin occurs in all effective nitrogen-fixing nodules in legumes, where it is essential for the facilitation of O_2 diffusion within the infected cells (Appleby, 1984). Hemoglobin is also found in the root nodules of *Parasponia*, a non-legume nodulated by *Rhizobium* (Appleby et al., 1983). In contrast, the degree of occurrence and function of hemoglobins in actinorhizal root nodules is uncertain.

Actinorhizal plants rival legumes as important nitrogen fixers in natural ecosystems but differ markedly from legumes in the physiology and anatomy of the root nodules (Schwintzer and Tjepkema, 1990). Little is known about hemoglobins in actinorhizal plants, but a hemoglobin has been found in high concentrations in the nodules of *Casuarina glauca*, and the purified protein has been extensively characterized and shown to be similar to the hemoglobins of legumes and *Parasponia* (Fleming et al., 1987). In addition, high concentrations of a hemoglobin-like protein have been found in nodules of *Casuarina cunninghamiana* and *Myrica gale* by using the 416- to 420-nm absorption band of carboxyhemoglobin to estimate the hemoglobin concentration (Tjepkema and Asa, 1987).

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Casuarina is unique among actinorhizal plants in that *Frankia*, the nitrogen-fixing microsymbiont, does not form vesicles within the nodule (Berg and McDowell, 1987). These vesicles are normally the site of nitrogen fixation and readily form in cultures of *Frankia* isolated from *Casuarina* and grown in the presence of moderate and high concentrations of O_2 (Murry et al., 1985). However, if cultures are grown at very low concentrations of O_2, vesicles do not form. This suggests that the hemoglobin found in *Casuarina* may be a special case related to the occurrence of very low O_2 concentrations in the nodules.

The formation of vesicles by *Frankia* in the root nodules of *Myrica gale* L. makes it uncertain whether zones of low O_2 occur in these nodules. Without zones of low O_2 concentration, the function of hemoglobin is unclear (Appleby, 1984), and it cannot be assumed that the hemoglobin-like protein found in these nodules is a true hemoglobin. The goal of the present study was to determine the nature of this hemoglobin-like protein by purifying it and comparing its properties with those of other hemoglobins. We found it to be a typical plant hemoglobin, except that it occurs as a dimer.

MATERIALS AND METHODS

Plant Growth

Seeds of *Myrica gale* L. were prechilled for 2 weeks at 5°C and germinated in light (100–300 μmol m^{-2} s^{-1} photosynthetic photon flux) on glass beads moistened with one-fourth-strength nitrogen-free Hoagland solution (Hoagland and Arnon, 1950) containing 1 mM urea. At 4 to 5 weeks after germination, seedlings were inoculated with *Frankia* strain LLR 161101 and grown in vermiculite in a greenhouse. Supplemental light (100–200 μmol m^{-2} s^{-1} photosynthetic photon flux) was provided in the winter. Plants were watered daily and fertilized three times a week with one-half-strength nitrogen-free Hoagland solution. Nodules were harvested at 12 to 14 weeks after inoculation.

Extraction of Hemoglobin

Hemoglobin was extracted from root nodules of *M. gale* by modifying the procedures used for *Parasponia* (Appleby et al., 1983), *Casuarina* (Fleming et al., 1987), and other actinorhizal plants (Tjepkema and Asa, 1987).

Abbreviations: V_e, elution volume; V_o, void volume.

Nodule roots were removed from fresh *M. gale* nodules and 0.3 to 0.7 g of nodules were added into 7 mL of CO-equilibrated extraction buffer in a 15-mL centrifuge tube (Corex; Corning Inc., Corning, NY). The extraction buffer contained 0.1 M potassium phosphate, 1 mM EDTA, 0.05% *n*-octyl β-D-glucopyranoside (Sigma), and 0.5 g of acid-washed polyvinylpolypyrrolidone (insoluble PVP; Sigma) dissolved in glass-distilled water and adjusted to pH 7.4. In preliminary experiments, the octyl glucoside was replaced by one of the following detergents: 0.01% Triton X-100 (Calbiochem, La Jolla, CA), 0.1% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (Sigma), or 0.1% Zwittergent 3–12 (Calbiochem). After flushing the headspace with CO for 60 s, 10 mg of sodium dithionite (Fluka) was added and the tube was sealed onto a Biospec (Bartlesville, OK) homogenizer (10,000 rpm; one-half-inch stator) using a rubber stopper. The headspace was flushed with nitrogen gas for an additional 60 s prior to homogenization at full speed for 2 min, and the centrifuge tube was immersed in an ice bath through the entire procedure. After homogenization, the tube was removed, stoppered, flushed with CO, and centrifuged at 4500*g* for 30 min at 4°C. The clear supernatant was carefully removed into a glass tube and equilibrated with CO, and the spectrum of a sample of this supernatant was recorded from 650 to 390 nm.

Gel Filtration

All chromatography was carried out in a cold room at 4°C. About 4 to 5 mL of the CO-saturated supernatant was pumped into a 73 × 1.6 cm column of Sephacryl S-200 HR (Pharmacia) equilibrated with degassed and CO-saturated buffer (glass-distilled water with 0.1 M potassium phosphate, 1 mM EDTA, pH 7.4). The column flow rate was 0.5 mL/min and 2-mL fractions were collected. The A_{280} protein peak was monitored (model 226 UV monitor, Instrumentation Specialties Co., Lincoln, NE) and the spectra of all protein fractions were recorded from 650 to 390 nm. The possible carboxyhemoglobin fractions were selected on the basis of the positions of the optical absorption peaks at 416 to 419 nm. The fractions were pooled, gently bubbled with CO, and pressure-filtered to a volume of about 1 mL over a Millipore (Bedford, MA) or Diaflo (Amicon, Beverly, MA) UM 10 membrane (M_r 10,000 exclusion) in an Amicon 50-mL concentration cell using nitrogen gas at 4°C.

Ion-Exchange Chromatography

The concentrated hemoglobin fractions in the pressure filtration cell were diluted with 2 mL of 0.01 M triethanolamine buffer (pH 7.5), degassed, and equilibrated with CO. Due to the partial oxidation of hemoglobin during pressure filtration, the sample was then reduced with a few crystals of sodium dithionite after equilibrating with CO in a sealed tube. This carboxyhemoglobin fraction was then pumped onto a 15 × 1.6 cm column of DEAE Sepharose CL-6B (Pharmacia) equilibrated with 0.01 M triethanolamine, pH 7.5, buffer at 4°C. The hemoglobin was visible as a red band at the top of the column. The column was then washed with 4 bed volumes of aerated 0.01 M triethanolamine buffer. Hemoglobin fractions were eluted (as oxyhemoglobin) using a linear gradient of 1.0 M potassium chloride in triethanolamine buffer at pH 7.5 and a flow rate of 0.5 mL/min. The A_{280} of each fraction was monitored and the oxyhemoglobin fractions were selected on the basis of the spectra from 650 to 390 nm.

Hemoglobin Spectra

Spectra were recorded from 650 to 390 nm at about 25°C using a Bausch and Lomb Spectronic 2000 spectrophotometer. Samples of 3.5 mL were used in a 4-cm semimicro, black-walled, glass cuvette. Passage of partially purified carboxyhemoglobin through the ion-exchange column converted it to pure oxyhemoglobin. Spectra were recorded either immediately after efflux from the column or after storage overnight at 3°C. To form ferrous hemoglobin, 4 mL of oxyhemoglobin was pipetted into a serum bottle (4 cm diameter) in an ice bath, flushed and equilibrated with argon, and reduced with a few crystals of sodium dithionite under a stream of argon. The spectrum was recorded immediately. To form carboxyhemoglobin, the deoxyhemoglobin in the cuvette was then very gently bubbled with CO for a few seconds.

Molecular Mass Determination by SDS-PAGE

Oxyhemoglobin eluted from the ion-exchange column was concentrated over Centricon microconcentrators (Amicon) and used for molecular mass determination by SDS-PAGE. Samples of 5, 10, 20, and 40 μL of *M. gale* hemoglobin were applied to four tracks on a 15% SDS-polyacrylamide slab gel cast against a polyacrylamide gel support medium (Gel Bond; FMC Bioproducts, Rockland, ME). Another track contained 10 μL of mixed Rainbow protein mol wt markers, mol wt range 14,300 to 200,000 (Amersham). The gel samples were prepared and run for 7 h according to the basic procedures of Laemmli (1970). The gel was fixed in a 25% isopropanol:10% acetic acid:2.5% glycerol mixture, stained for 20 min with 0.05% Coomassie blue dissolved in the same fixative solution, and destained with 40% methanol:10% acetic acid:2.5% glycerol solution until optimum resolution was obtained.

Mol Wt Determination by Gel Chromatography

The Sephacryl S-200 HR gel column used for the purification of carboxyhemoglobin was also used for mol wt determination. Samples of 4 mL of *M. gale* hemoglobin extract and mol wt markers (BSA, 66,000; chymotrypsinogen A, 25,000; lactoglobulin A, 36,500; and RNase A, 13,700; all from Sigma) were individually passed through the column at 0.5 mL/min and 2-mL fractions were collected. The V_e was determined by measuring the A_{280} except in the case of *M. gale* hemoglobin, where the 416.2-nm absorbance of carboxyhemoglobin was used. The V_o was determined by running a 4-mL sample of 2 mg/mL blue dextran under identical conditions. The molecular mass of *M. gale* hemo-

globin was calculated from a plot of V_e/V_o against log (mol wt) (Whitaker, 1963).

RESULTS AND DISCUSSION

A stable hemoglobin was extracted from the root nodules of *M. gale* L. by the procedure described above. A detergent was necessary to extract significant quantities of hemoglobin. A very low but measurable quantity of hemoglobin could be extracted even when no detergent was used in the medium. In preliminary experiments, we compared the yields of hemoglobin with various detergents included singly in the extraction medium. Both 0.01% Triton X-100 and 0.05% octyl glucopyranoside gave yields comparable to those found previously using 1% Triton X-100 (Tjepkema and Asa, 1987), whereas 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate and Zwittergent 3–12 resulted in a somewhat lower yield. In subsequent experiments, octyl glucoside was used because it is less likely than other detergents to damage proteins and can be easily removed during protein purification because of its high critical micelle concentration (Chattopadhyay and London, 1984). In previous work with *Casuarina* (Fleming et al., 1987), soluble PVP was included in the extraction buffer, but in *M. gale*, we obtained higher yields of hemoglobin when a detergent was used instead.

The amount of phenolics in the extract, as judged by *A* in the 390- to 450-nm wavelength region, was reduced by the use of insoluble PVP during extraction. We also tried XAD-4 resin beads (Serva, Paramus, NJ) for this purpose, but the recovery was reduced due to binding of hemoglobin to the resin. In some experiments, we substituted 5 mM DTT (Calbiochem) for the sodium dithionite in the extraction medium. The yields of hemoglobin were similar, but there was greater background *A* in the 390- to 450-nm wavelength region.

It was necessary to use conditions favoring high resolution to separate the main hemoglobin component by gel filtration. The hemoglobin was preceded in elution by high mol wt compounds (phenolics?) that began eluting at about the V_o of the column and had spectra with a steep rise in *A* between 450 and 390 nm. After the main hemoglobin peak, there was a second peak of another possible hemoglobin (Fig. 1).

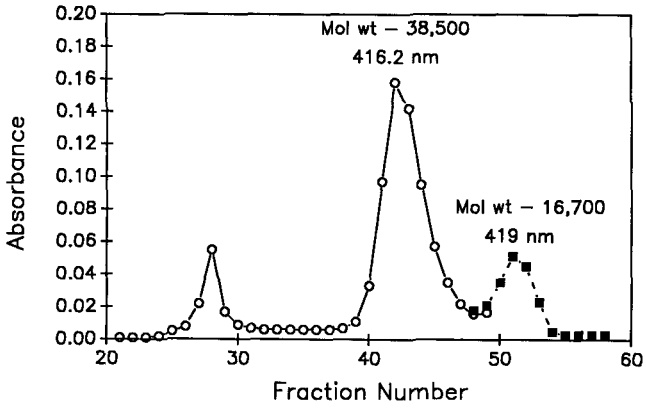

Figure 1. Chromatography of crude *M. gale* carboxyhemoglobin on a Sephacryl S-200 HR column as described in the text. Fractions 21 to 49 were monitored at 416.2 nm (○) and fractions 48 to 58 were monitored at 419 nm (■). This was done because the peak *A* for fraction 42 was at 416.2 nm, whereas for fraction 51 the peak *A* was at 419 nm.

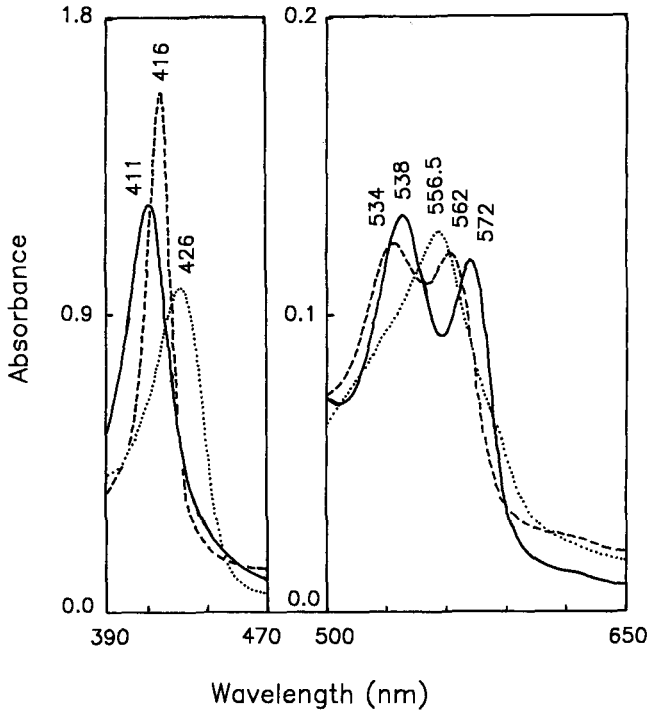

Figure 2. Optical absorption spectra of purified *M. gale* ferrous hemoglobin (· · ·), ferrous oxyhemoglobin (—), and ferrous carboxyhemoglobin (- - - -). Different *A* scales were used in the right and left panels because of the large difference in hemoglobin *A* in these two wavelength regions.

Various attempts were made to form oxyhemoglobin from the carboxyhemoglobin eluted from the gel column. However, these were not successful and instead resulted in the formation of ferric hemoglobin. Partial oxidation to the ferric form also occurred during concentration of the gel-purified hemoglobin by ultrafiltration. We overcame this problem by further purification of hemoglobin extracts by ion exchange using air-equilibrated buffer. The hemoglobin was eluted in a stable oxygenated form in a tight red band at a potassium chloride concentration of about 0.2 M. This purified hemoglobin could be stored for at least 1 d at 5°C without appreciable oxidation to the ferric form.

Absorption spectra of the deoxy-, oxy-, and carboxy-forms in both the 411- to 426-nm and the 534- to 572-nm wavelength regions were very similar to those obtained previously for hemoglobins from *Casuarina* (Fleming et al., 1987), *Parasponia* (Appleby et al., 1983), and various legume nodules (Appleby, 1984) (Fig. 2).

A high degree of purity was obtained by the combination of gel filtration and ion exchange. The ratio of A_{280} (protein):A_{416} (carboxyhemoglobin) was 3.2, which is comparable to that observed for hemoglobin purified from *Casu-*

arina (Fleming et al., 1987). Using SDS gel electrophoresis, only a single band was observed at the expected position for a monomeric hemoglobin.

The molecular mass estimated by SDS-PAGE was 16,200 D (Fig. 3). The molecular mass was also estimated by gel filtration of the purified hemoglobin with the same column used during the initial purification. The hemoglobin eluted in exactly the same fraction as before purification, at an estimated molecular mass of 38,500 D (Fig. 4). Thus, the hemoglobin appears to occur as a dimer, as previously observed for the hemoglobin from *Parasponia* nodules (Appleby et al., 1983).

We are uncertain about the nature of the second hemoglobin-like compound observed after the main hemoglobin peak (Fig. 1). The relative proportions of the two peaks were very consistent between different preparations. The molecular mass of this second protein was about 16,700 D (Fig. 4) and is consistent with that of a monomeric hemoglobin. The absorption spectra of the CO form is also consistent with that of hemoglobin. We have not excluded the possibility that this protein is simply a monomeric form of the major hemoglobin component. However, the absorption maximum in the Soret region is at 419 nm rather than at the 416.2 nm observed for the dimer.

The most probable function for the hemoglobin in the nodules of *M. gale* is in facilitation of O_2 transport. Zones of low O_2 concentration have been shown within these nodules (Tjepkema, 1983a), and the concentration of hemoglobin is sufficient to allow a high rate of O_2 transport. Spectra of nodule slices also show a rapid interconversion between the oxygenated and deoxygenated forms of hemoglobin in atmospheres of pure N_2 and O_2 (Tjepkema, 1983b). Nonetheless, the formation of vesicles by *Frankia* in *Myrica* nodules suggests a higher O_2 concentration than in the nodules of *Casuarina* (see the introduction). A hemoglobin function other than O_2 transport cannot be ruled out (Wittenberg and Wittenberg, 1990), and the occurrence of hemoglobins in nonnodulated plants (Appleby et al., 1988; Taylor et al., 1994) also raises this possibility.

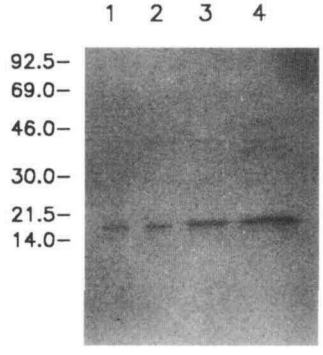

Figure 3. Estimation of the molecular mass of *M. gale* hemoglobin by SDS-PAGE. Molecular masses of calibration proteins are indicated in kD. Lanes 1 through 4 contain 5, 10, 20, and 40 μL of purified hemoglobin, respectively. The estimated mass was 16,200 D.

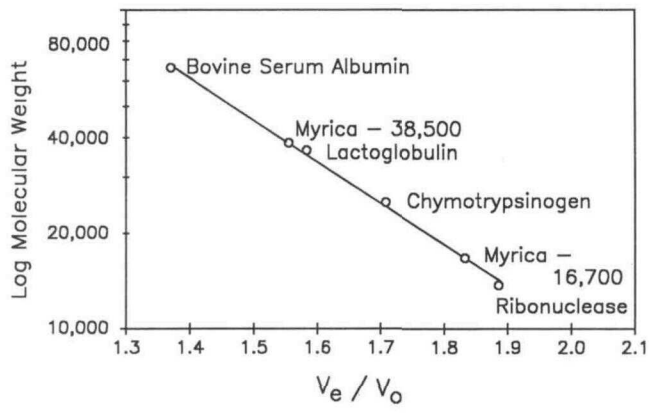

Figure 4. Estimation of the mol wt of *M. gale* hemoglobin by gel filtration on a Sephacryl S-200 column. After the mol wt standards were plotted, the mol wts of the two *M. gale* hemoglobins were obtained from the intersection of their V_e/V_o values with the plot.

CONCLUSIONS

Our results support the suggestion that the hemoglobin-like protein found in the nodules of *M. gale* (Tjepkema and Asa, 1987) is a true hemoglobin. No other hemoprotein has been reported that forms a stable oxygen complex or has a subunit molecular mass of 16,000 to 19,000 D. The absorption spectra of the oxy-, deoxy-, and carboxy- forms are almost identical to those of purified hemoglobins from nodules of soybean and *Parasponia* (Appleby et al., 1983). As reported previously, the concentration of hemoprotein in the nodule is very similar to that found in the nodules of legumes, *Parasponia*, and *Casuarina*. As in the case of *Parasponia*, the hemoglobin is a dimer.

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